Songklanakarin J. Sci. Technol.
44 (1), 97-102, Jan. - Feb. 2022

http://www.sjst.psu.ac.th

Original Article

Types of cells in the hepatopancreas of the Pacific whiteleg shrimp *Litopenaeus vannamei* being infected by *Enterocytozoon hepatopenaei*♣

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Received: 30 March 2021; Revised: 18 August 2021; Accepted: 22 August 2021

Abstract

Enterocytozoon hepatopenaei (EHP), the fungus-related micro-organism that infects the hepatopancreas of marine shrimp, is believed to cause growth retardation of the infected shrimp. As the hepatopancreatic cells are composed of different cell types that support the digestive system and innate defense, malfunction of the infected cells could lead to growth retardation and weakness. This study aimed to determine of the percentage of each hepatopancreatic cell type infected by EHP and its infectious levels in the normal and growth-retarded Pacific whiteleg shrimp *Litopenaeus vannamei*. Histological examination revealed that all the cell types examined were infected with EHP, with significantly higher ($p<0.01$) percentage on the M- and F-cells, compared to other cell types. The R-, B- and F-cells of the growth-retarded shrimp were infected at significantly lower ($p<0.05$) percentage than the same cell types of the normal shrimp. By PCR, positive reactions of EHP infection were detected in all shrimp samples, both in the normal and growth-retarded shrimp, but only at the nested PCR level. Also, the relative density of the EHP load in the hepatopancreas of the two shrimp groups did not differ statistically. These results suggest that growth retardation in *L. vannamei* currently exists may not be due to EHP infection.

Keywords: growth retardation, white-feces disease, shrimp culture, *Enterocytozoon hepatopenaei*, Microsporidia

1. Introduction

Among several problems plaguing the marine shrimp industry in Thailand, the infection by *Enterocytozoon hepatopenaei* (EHP) has severely damaged the production of the Pacific whiteleg shrimp *Litopenaeus vannamei*. The pathogen, EHP, is a fungus-related micro-organism belonging to the phylum Microsporidia based on its DNA sequence (Vávra & Lukeš, 2013; Vossbrinck & Debrunner-Vossbrinck, 2005). It is believed that shrimp being infected by EHP have retarded growth and the condition is associated with white-feces disease (Tang *et al*., 2016), leading to chronic morbidity and mortality.

EHP infection in marine shrimp was first reported in the black tiger shrimp, *Penaeus monodon* (Anderson, Shariff, & Nash, 1989) in Malaysia, followed by the Kuruma shrimp, *Marsupenaeus japonicus* (Hudson, Hudson & Pyecroft, 2001) in Australia, and again in *P. monodon* suffering from the slow-growth syndrome in Thailand (Chayaburakul, Nash, Pratanpipat, Sriurairatana, & Withyachumnarnkul, 2004; Tourtip *et al*., 2009). In the 1990s, when the majority of Thai

♣Peer-reviewed paper selected from The 1st International Conference on Sustainable Agriculture and Aquaculture (ICSAA-2021)
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shrimp growers switched from growing *P. monodon* to *L. vannamei*, it was reported that *L. vannamei* was also susceptible to EHP infection, and the prevalence at present has been increasing to the point that it is difficult to find any individual shrimp in the grow-out farms that is not infected by the pathogen (https://www.speedyassay.com/ehp-and-ems-cases-on-the-rise-in-southeast-asian-shrimp-farms-2/). It is not clear how the infection is so widely spread. Positive detections of EHP by polymerase chain reaction (PCR) method of sediments in public waterways of several locations suggest the pandemic presence of the pathogen (unpublished data). EHP is a spore-forming pathogen and the spores can stay for a long, but unknown, period if not destroyed by disinfectants, including potassium permanganate (15 ppm), chlorine (40 ppm for 15 min or 10 ppm for 24 h), 20% ethanol (15 min), or by raising the water pH above 11 (Aldama-Cano *et al*., 2018; Chaijarasphong *et al*., 2020). Several aquatic animals, such as fish, mollusks, *Artemia*, and copepods have been found to be PCR-positive for EHP although it is not known if these animals are mechanical carriers or being biologically infected by the pathogen (unpublished data).

Severe EHP infection may be associated with white-feces disease, followed by chronic morbidity and mortality of the shrimp (Sriurairatana *et al*., 2014). At the present situation, what shrimp growers in Thailand could do is stock EHP-free fry and treat supplying water with either chlorine or potassium permanganate in the reservoir, as well as treating pond bottom with the disinfectant(s) as recommended (Aldama-Cano *et al*., 2018).

In shrimp, hepatopancreas and the front part of the foregut epithelium were found to be the only host cells of EHP (Chaijarasphong *et al*., 2020). The hepatopancreas of marine shrimp is on elaborate tubular structures consisting of at least five cell types: E (embryonic), M (midget or basal), F (fibrillar), B (blister or vesicular), and R (reabsorption or reserve) cells (Hu & Leung, 2007; Nunes, Braga, & Camargo-Mathias, 2014). While the functions of all these cell types have not been fully elucidated, their provisional functions have been described based on limited scientific evidence. The E-cells are the non-specialized cells that divide mitotically to replace other cell types that are worn out. The M-cells are located at the basal part of the hepatopancreatic epithelium and have no direct contact with the hepatopancreatic lumen (Nunes *et al*., 2014). The F-cells synthesize digestive enzymes, which are stored in a single large vesicle, and become B-cell (Hu & Leung, 2007). The B-cells release the digestive enzymes into the hepatopancreatic lumen through the holocrine secretion process. The R-cells have multiple small vesicles containing digested nutrients absorbed from the hepatopancreatic lumen; thus, they are nutrient storage cells (Nunes *et al*., 2014).

Although it is believed that growth retardation of the EHP-infected shrimp is caused by the infection of these cell types, interfering with the shrimp nutritional status, it has not been shown what cell types are infected, or at any different rate. Also, the belief that growth retardation is positively correlated to the severity of EHP infection, has not been proven scientifically. Therefore, this study aimed at the determination of the cell types of EHP infection in the hepatopancreas of *L. vannamei* and attempted to find a correlation between the severity of EHP infection and the growth rate of the shrimp.

2. Materials and Methods

Approximately 200 *L. vannamei* individuals were randomly collected by net casting at day 90 of culture (DOC) from a commercial shrimp pond being considered to have a growth retardation phenomenon. The pond, 1.0-hectare size, was stocked with specific pathogen-free *L. vannamei* postlarvae at the stocking density of 100 individuals/m^3 of 30ppt seawater. The shrimp were provided with commercial feed pellets, composed of 35% protein. With that level of stocking density, the average daily growth (ADG) of the shrimp being considered normal should be at any level higher than 0.2 g per day. However, the ADG of the shrimp randomly sampled from the pond calculated from the first day of the stocking to DOC 90 was 0.12 g per day and the coefficient of variation (CV) of the shrimp body weights (BW) was 35.64%; these two parameters were an indication that the shrimp in the pond had growth retardation and individual size difference (Sriurairatana *et al*., 2014; Tourtip *et al*., 2009).

The shrimp were divided into normal and growth-retarded groups based on their body weight (BW), with 15-20g BW for normal and 5-10g BW for growth-retarded shrimp. The shrimp, 20 individuals per group, were individually weighed before their hepatopancreas was carefully isolated and weighed. The hepatopancreas was roughly divided into two parts: one was fixed in Davidson's fixative for paraffin sectioning and another in 95% ethanol for PCR determination. For histological examination, 6 samples of hepatopancreas from each group of shrimp were processed. For PCR, 10 samples from each group of shrimp were determined.

The Davidson-fixed tissue was routinely processed through the standard paraffin sectioning method, by being dehydrated with the series of ethanol, infiltrated and embedded in paraffin, sectioned at 6 µm thickness, and stained with hematoxylin and eosin (H&E). The sections were examined through the 100x objective lens of the light microscope. Hepatopancreatic epithelial cells, M-, R-, B- and F-cells, were counted, either as EHP-infected or non-infected. The infected cells were identified by the presence of EHP spores or plasmodium in the cytoplasm. The proximal and middle regions, but not the distal ones, of the hepatopancreas were examined since these two regions contain various cell types at similar proportions, whereas the distal region comprises a high proportion of E-cells (Hu & Leung, 2007; Nunes *et al*., 2014). The counts were performed on cells belonging to different cell types under the same microscopic field, and different microscopic fields were randomly selected until at least 200 cells of each cell type were counted for an individual shrimp, which were distributed in approximately 50 tubules.

The detection of EHP by PCR was performed according to the method previously described (Jaroenlak *et al*., 2016). Briefly, the 95% ethanol-fixed hepatopancreas was homogenized and incubated in 500 µL lysis buffer containing 5 µg/mL proteinase K, and extracted for DNA by phenol-chloroform and treated with DNase-free RNase (New England Biolabs, USA). The concentration of the extracted DNA was determined using a NanoDrop Spectrophotometer (Thermo Scientific, USA).

The amplification process for the transcript encoding the spore wall protein (SWP) of EHP was performed in the thermal cycler by employing 2 sets of primers (Table 1): SWP_1F and SWP_1R primers for the first-step and SWP_2F and SWP_2R primers for the nested-step PCR. The primers used for the internal control, the actin gene, were Actin_F and Actin_R. The PCR reaction mixture (25 µL) was composed of 2 µL DNA template (25 ng/µL), 5 µL 5x HOT FIREPOL® Blend DNA Polymerase (10 mM $MgCl_2$), 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM) and 17 µL ddH_2O. The PCR reaction was run for 30 cycles comprising of denaturation at 95 ºC for 30 s, annealing at 58 ºC for 30 s, and extension at 68 ºC for 45 s with a final 5-min extension step at 68 ºC.

The difference between the first and the nested-step PCR was that in the nested-PCR step, the DNA template was 1.0 µL of the PCR product obtained from the first-step and its PCR condition was also different, i.e., annealing at 64 ºC for 30 s, and extension at 68 ºC for 20 s and was run for 20 cycles. The actin PCR condition was the same as the first-step SWP PCR, except for the 55 ºC annealing temperature.

The amplicons were analyzed by 1.5% agarose gel electrophoresis with SYBER safe staining, using DNA ladder markers (2 logs, 100 bp, or 1 kb DNA ladder from New England Biolabs, USA). The PCR bands were visualized under UV illuminator and semi-quantitated by standardized with the actin band, using the program ImageJ.

The numerical data were expressed as means ± SD (N) and statistical analysis was performed by ANOVA followed by Tukey's test, and by Student's t-test. A significant difference was noted when $p<0.05$.

3. Results and Discussion

The average BW of the normal shrimp was 17.6 ± 1.6 (20) g and that of growth-retarded ones was 8.7 ± 1.1 (20) g; the two values were significantly different by statistical comparison ($p<0.01$). The absolute weight of the hepatopancreas of the normal shrimp was significantly larger ($p<0.01$) than that of the growth-retarded shrimp, but its relative weight when being standardized by BW did not differ statistically (Figure 1).

By histology, R-cells constituted the highest percentage among the four types of cells under study, which was more than 70%; the percentage was significantly ($p<0.01$) higher than that of other cell types (Table 2). The second most frequent cell detected were B-cells, which constituted around 15%, while the other two types, M- and F-cells were <10%, and F-cells constituted the lowest percentage among all cell types, which was significantly lower than that of B-cells ($p<0.01$), but not significantly different from that of M-cells. This pattern of difference in the percentage of cell types, R>B>F=M, was also observed in the growth-retarded shrimp. When comparing the percentage of any particular cell types of the normal and the growth-retarded shrimp, no statistical significance was detected.

To our knowledge, the percentage of hepatopancreatic cell types for *L. vannamei* has not been reported. In the giant freshwater prawn *M. rosenbergii*, R- and B-cells shared the highest percentage of cells found in the hepatopancreas (Silva, Almeida Neto, Ramiro, Santos, & Guerra, 2018), which was approximately 30-40% each. The discrepancy between their and our reports, if not considering the difference in species, might be due to the different criteria for the identifications of R- and B-cells. In our study, B-cells were identified as cells containing a single large vesicle and R-cells as the cell containing multiple small vesicles; while Silva *et al*. (2018) had identified the B-cells as the cells containing both large and small vesicles. In marine shrimp, it has been well accepted that B-cells contain one large vesicles, while R-cells contain multiple small vesicles (Nunes *et al*., 2014).

At present, the function of R-cells is still not clear. Its numerous small vacuoles in the cytoplasm contain lipid and glycogen (Nunes *et al*., 2014), therefore they are regarded as nutrient storage cells. In that sense, it would be similar to the liver cells of higher animals that function as glycogen reserve for glucose utilization. The shrimp may use both glycogen and lipid as the reserved nutrients. The finding that the percentage of R-cells in the hepatopancreas was the same in both the normal and growth-retarded shrimp suggested that growth retardation, in this case, might not be due to the inadequate nutritional reserve. An argument on the possible low absolute number of R-cells in the hepatopancreas despite the normal percentage of the cells is unlikely since the relative weight of the hepatopancreas of the two groups of shrimp was comparable.

Histology of the four cell types, normal and EHP-infected, is shown in Figure 2. The EHP-infected cells contain either plasmodium, which appeared as basophilic nodules, or spores, which appeared as eosinophilic granules, in the cytoplasm. In the determination of the percentage of each cell type being infected by EHP, it revealed that M- and F-cells were infected at a significantly higher rate ($p<0.01$) than R- and B-cells, and R-cells were infected at significantly higher rate ($p<0.01$) than B-cells, i.e., with the pattern M=F>R>B

Table 1. Nucleotide sequences of primers used to amplify SWP transcript of EHP and actin gene for the internal control, as described by Jaroenlak *et al*. (2016)

Primer	Sequence (5' to 3')	Product size (bp)
First-step		
SWP_1F	TTGCAGAGTGTTGTTAAGGGTTT	514
SWP_1R	CACGATGTGTCTTTGCAATTTTC	
Nested step		
SWP_2F	TTGGCGGCACAATTCTCAAACA	148
SWP_2R	GCTGTTTGTCTCCAACTGTATTTGA	
Actin gene		
Actin_F	CCTCGCTGGAGAAGTCCTAC	401
Actin_R	TGGTCCAGACTCGTCGTACTC	

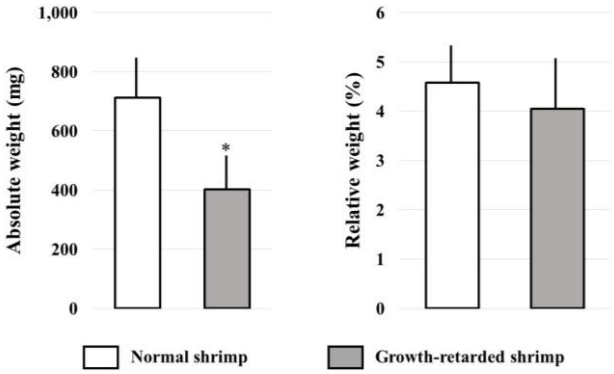

Figure 1. The absolute and relative weight of the hepatopancreas of the normal and growth-retarded Pacific whiteleg shrimp *Litopenaeus vannamei*. *p<0.05

(Table 2). Similar pattern was also observed in the growth-retarded shrimp, but the prevalence in R-cells was comparable to that of B-cells, i.e., the pattern became M=F>R=B. When comparing the percentage of EHP infection in any cell type between the normal and growth-retarded shrimp, it was surprising to find that the cells of the growth-retarded shrimp were infected at a lower percentage than those of the normal shrimp. A significantly lower percentage of EHP infection in the growth-retarded shrimp was found in the R-cells ($p<0.01$), B-cells ($p<0.05$), and F-cells ($p<0.05$).

It is interesting that M-cells, which constituted only 6-7% of the total number of cells under study, were infected by EHP at the highest rate, i.e., at 75-85% (Table 2). By its basophilic cytoplasm, its cytoplasm likely contains high concentrations of various types of acidic substances, including mRNA, rRNA, and tRNA, which are important for the protein translation process. In that case, M-cells may be highly active in protein synthesis. The shrimp hepatopancreas has been

Table 2. Percentage of cell types, the prevalence of EHP-infected cells, and relative density of EHP infection in the hepatopancreas of the normal and growth-retarded *L. vannamei*. Different superscripts indicate that the difference among different cell types within the same group of animal reaches statistical significance.

Cell types in the hepatopancreas (%)

	M-cell	R-cell	B-cell	F-cell
Normal	6.7 ± 3.1 (6)^a	73.9 ± 7.3 (6)^b	14.0 ± 4.7 (6)^c	5.3 ± 1.6 (6)^a
Growth-retarded	6.4 ± 2.2 (6)^a	75.7 ± 4.2 (6)^b	13.5 ± 3.7 (6)^c	4.4 ± 0.6 (6)^a

Prevalence of EHP-infected cells (%)

	M-cell	R-cell	B-cell	F-cell
Normal	86.3 ± 7.1 (6)^a	47.5 ± 15.6 (6)^b	22.2 ± 10.2 (6)^c	84.9 ± 7.4 (6)^a
Growth-retarded	76.7 ± 10.9 (6)^a	15.6 ± 13.0 (6)^b	6.6 ± 6.3 (6)^b	65.9 ± 18.5 (6)^a

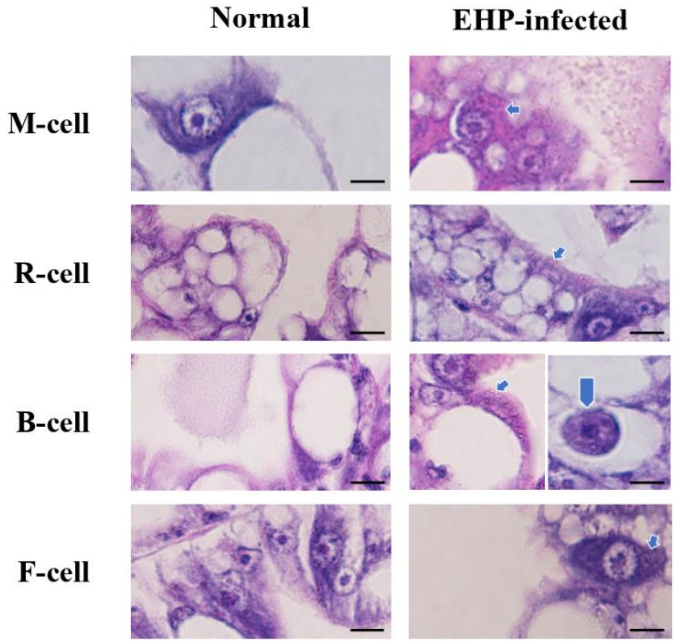

Figure 2. Histology of the normal and EHP-infected cells of the four cell types in the hepatopancreas of *L. vannamei* in this study, showing EHP spores in all cell types (arrows) and EHP plasmodium in the B-cell (arrowhead). H&E staining. The length of the bar in each picture equals 10 µm

known to produce hemocyanin, vitellogenin, and other important proteins to guard against pathogens, like anti-microbial peptides and heat-shock proteins (Tseng *et al*., 2002; Zhang, Huang, & Qin, 2004; Yang Huang, Wang, Aweya, Zheng, & Zhang, 2018; Tassanakajon, Amparyup, Somboonwiwat, & Supungul, 2011). High prevalence of EHP infection in this cell type might reduce the efficiency of M-cells to produce these proteins and lead to reduce respiratory ability (by reducing hemocyanin level) and innate defense, which could lead to superimposed bacterial infection, as has become the hypothesis for white-feces disease in the shrimp (Sriurairatana *et al*., 2014).

Vossbrinck and Debrunner-Vossbrinck (2005) reported that EHP had no mitochondria and, thus, cannot produce ATP; the pathogen takes ATP as an energy source from the host cell for its benefit. The finding in this study that M- and F-cells were infected by EHP at the highest rate, compared to other cell types, is probably due to a possibility that EHP thrives well in these two types of cells that have active protein production, and thus, contain a high amount of ATP.

The detection of EHP in the hepatopancreas samples of the normal and growth-retarded shrimp by PCR revealed that all the samples in both groups of shrimp were positive, but only in nested-step PCR, suggesting that all the shrimp were lightly infected by the pathogen (Figure 3). When the density of the amplicon bands on the gel was semi-quantified by ImageJ program and standardized by the actin band, it showed a high variation of the levels of EHP infection in the growth-retarded shrimp, with the average level (0.57 ± 0.52) higher than, but non-significantly different from, that of the normal shrimp (0.32 ± 0.12).

If EHP infection directly causes growth retardation in the shrimp, the growth-retarded shrimp would have a higher load of EHP in the hepatopancreas than in the normal shrimp. The finding in this study, although showing that the growth-retarded shrimp had a higher EHP load than that of the normal shrimp, the difference did not reach statistical significance. It implies that both groups of shrimp had a comparable load of EHP in the hepatopancreas. Together with the findings that all the cell types in the hepatopancreas of the growth-retarded shrimp had a lower prevalence of EHP infection than that of the normal shrimp suggested that EHP infection may not be related to shrimp growth, at least in the light level of infection as in this study. These data were surprisingly similar to that reported earlier by Rajendran *et al*. (2016), in which the growth-retarded *L. vannamei* had a much lower prevalence of EHP infection than that of the normal shrimp. Other unknown factors, probably variation in the shrimp genome within a single population, may play a more important role in the growth rate than the EHP infection.

4. Conclusions

EHP infects all the specialized cells of the hepatopancreas of *L. vannamei*, and M- and F-cells were infected at a higher percentage than the R- and B-cells. A possible explanation is that M- and F-cells are highly active in protein synthesis and most likely contain high levels of ATP, the energy compound needed by EHP. The equal EHP load in the hepatopancreas of the normal and growth-retarded shrimp in this study does not support the hypothesis that growth retardation of the shrimp is directly related to the intensity of the infection. It should be noted that EHP infection in this study was at light, nested PCR-positive, level. Under heavy infection, e.g., at first-step PCR-positive, the correlation between the intensity of infection and growth retardation may be observed. Besides, the levels of virus load, rather than the percentage of infected cells in any particular cell type, may be more influential in causing the growth retardation than the overall severity of the infection. All these questions await future research.

Acknowledgements

The study was funded by the Prince of Songkla University, we would like to thank the Discipline of Excellence for Sustainable Aquaculture, Prince of Songkla University for financial support, 2019-20.

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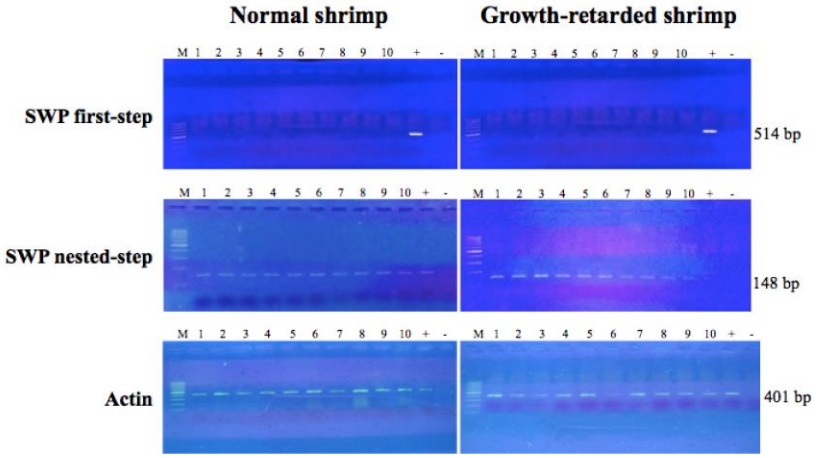

Figure 3. Photograph of the gel electrophoresis of the amplicons from PCR reactions using SWP- and actin-specific primers of the normal and growth-retarded Pacific whiteleg shrimp *Litopenaeus vannamei*

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